The murine placenta contains hematopoietic stem cells within the vascular labyrinth region

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The Murine Placenta Contains Hematopoietic Stem Cells within the Vascular Labyrinth Region

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Summary

In the midgestation murine embryo, several major vascular tissues contain hematopoietic stem cell (HSC) activity. These include the aorta-gonad-mesonephros region (AGM), yolk sac, and fetal liver. Recently, the placenta was demonstrated to harbor hematopoietic progenitors, but it was not examined for HSC activity. We demonstrate here that the placenta also harbors adult-repopulating HSCs. Placental HSCs begin to be detected at embryonic day (E) 11, and HSC numbers increase dramatically between E11 and E12, exceeding the numbers in the circulating embryonic blood. Furthermore, all placental HSC activity is restricted to the GFP$^+$ fraction of cells in *Ly-6A* (Sca-1) *GFP* transgenic embryos. Cells coexpressing GFP and endothelial markers CD34 and CD31 are found in the embryonic vasculature of the placental labyrinth. Moreover, placental cell expression of other HSC markers and transcription factors suggests that HSC emergence may occur in the placenta, as has been proposed for other embryonic hematopoietic sites.

Introduction

For over 30 years, there has been a continuing debate concerning the origin of definitive hematopoiesis in the murine embryo. As the yolk sac (YS) is the first tissue during development to produce blood cells (primitive erythroid cells), it was assumed to be the sole source of hematopoiesis in the mammalian embryo (Moore and Metcalf, 1970). However, accumulating evidence pointed to another anatomical site within the embryo as the source of definitive hematopoietic cells. Avian and amphibian embryo grafting experiments demonstrated that the adult hematopoietic system was derived primarily from the embryonic part of the conceptus in a mesodermal region containing the dorsal aorta and pronephros (Chen and Turpen, 1995; Dieterlen-Lievre, 1975). More recent experiments in amphibian embryos have confirmed these results and further demonstrate that distinct blastomeres in the 32-cell stage give rise to hematopoietic cells of the YS and embryo body (Ciau-Uitz et al., 2000). In the mouse, it was more recently shown that the first hematopoietic stem cells (HSCs; cells fully competent to long-term reconstitute the entire adult hematopoietic system after transplantation into irradiated, adult recipients) are autonomously generated in the intraembryonic aorta-gonad-mesonephros (AGM) region, and more precisely the aorta-mesenchyme subregion at embryonic day (E) 10.5 (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). While this is the first source of such fully competent HSCs, other in vivo transplantation scenarios (injection into the placenta or YS cavity of embryos, injection into the liver of conditioned neonatal recipients, or intravenous injection into immunodeficient recipients) have revealed long-term repopulating cells with multipotential hematopoietic properties at earlier embryonic stages (Cumano et al., 2001; Toles et al., 1989; Weissman et al., 1978; Yoder et al., 1997a, 1997b). Such multipotential cells were found in the YS and paraaortic splanchnopleura (precursor tissue to the AGM) at E8, E9, and E10. Hence, both the YS and intraembryonic tissues are capable of generating hematopoietic cells, and it is as yet undetermined whether cross-seeding of hematopoietic tissues occurs via the circulation and whether tissue-specific maturation of hematopoietic cells is required for the generation of the adult hematopoietic system. Importantly, a recent detailed quantitative analysis of HSC distribution in the midgestation embryo suggests that the fetal liver is colonized from multiple sources, including the AGM and YS (Kumaravelu et al., 2002).

In addition to the AGM and the YS, the allantois has been identified as a third site of hematopoiesis in the chick embryo (Caprioli et al., 1998). However, in murine embryos, it is not yet clear whether the allantois is hematopoietic (Downs and Harmann, 1997). The murine allantois derives from the mesoderm at the posterior end of the mouse embryo and makes contact with the chorion at E8.5 (reviewed in Cross, 1998; Cross et al., 2003a, 2003b; Han and Carter, 2001; Rossant and Cross, 2001). The chorioallantoic placenta forms at this junction, and, thereafter, the allantois contributes the fetal vascular and associated stromal components, including the umbilical vessels (Downs et al., 1998). Interestingly, the placenta was recently demonstrated to contain clonogenic hematopoietic progenitors, including CFU-GMs, CFU-GEMMs, BFU-Es, and HPP-CFCs (Alvarez-Silva et al., 2003). Moreover, between E10 and E12, these progenitors were present at numbers higher than those found in the YS and fetal liver. However, it was not investigated whether the placenta also contains HSCs.

A well-recognized feature shared by embryonic hematopoietic sites is the associated development of the vascular and hematopoietic systems. A wide range of evidence has supported hemangioblasts and hemogenic endothelium as the presumptive precursors to emerging hematopoietic cells (reviewed in Dieterlen-Lievre, 1998; Nishikawa, 2001; Ottersbach and Dzierzak, 2005; see http://Eurekah.com for the online version of this chapter). In *Ly-6A GFP* transgenic mice expressing the green fluorescent protein (GFP) under the regulatory elements of the HSC marker Sca-1, we demonstrated that all HSC activity was confined to the GFP$^+$ fraction in AGM, fetal liver, and adult bone marrow tissues (de Bruijn et al., 2002; Ma et al., 2002). We further showed by immunohistologic analysis of E9–E11 em-

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bryos that GFP expression localized to the endothelial layer of the dorsal aorta, the vitelline artery, and the major blood vessels of the YS. The common expression of this transgene, as well as other markers, in endothelial cells and AGM HSCs highlights the close relationship between these two cell types and supports the notion that HSCs derive from specialized "hemogenic" endothelial cells that are present for a brief period of time during midgestation.

In extending our analysis of these HSC marker transgenic embryos to earlier times in development, E6–E7.5, we found high GFP expression restricted to extraembryonic tissues: the ectoplacental cone and the extraembryonic ectoderm. Since these sites contribute to the placenta, the temporal expression was examinedin the placenta, beginning from E9, just after the fusion of the chorion and the allantois. At all stages examined, we found high-level GFP expression restricted to the embryonic/fetal vessels of the placental labyrinth (the layer of the placenta in which the embryonic circulation comes into contact with the maternal circulation). This observation prompted us to test for HSC activity in the placenta. We show here that adult-type HSCs are indeed present in the placenta, starting from E11, and that they are again exclusively found in the Ly-6A GFP$^+$ fraction. Moreover, other HSC markers colocalize with GFP$^+$ cells in the labyrinth region. These results suggest that the placenta, together with other distinct embryonic tissues, contribute to the adult hematopietic system.

Results

Expression of Ly-6A GFP in the Highly Vascularized Tissues of the Embryo

Previously, we have shown that all HSCs in the AGM region of the midgestation mouse embryo are localized to the Ly-6A GFP$^+$ aortic fraction of cells (de Bruijn et al., 2002). GFP expression is found in some endothelial cells of the dorsal aorta, and an even stronger expression is found in the endothelial cells lining the vitelline artery (Figure 1A). Hematopoietic clusters along the lumen of the aorta and vitelline arteries also contain GFP$^+$ cells (Figure 1B). Moreover, strong GFP expression is found in the umbilical artery (Figure 1E) and the major vessels of the YS (de Bruijn et al., 2002). All these sites contain potent HSC activity as determined by in vivo transplantation into adult, irradiated recipients (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996).

In addition to these known hematopoietic tissues, the placenta is also highly vascularized, and, thus, we examined this tissue for expression of the Ly-6A GFP marker. As the placenta is formed from both maternally and embryonic-derived cells, the *Ly-6A GFP* transgene was transmitted only through the male germline. Already in the prestreak-stage embryo, high GFP expression is also found in the extraembryonic ectoderm and ectoplacental cone (Figures 1C and 1D). The highest intensity of GFP expression is found where the extraembryonic ectoderm borders the primitive ectoderm. At E7.25, the extraembryonic ectoderm remains GFP$^+$ (Figures 1F–1H). At this stage, the exocoelom is forming and the extraembryonic ectoderm appears to be advancing toward the GFP$^+$ ectoplacental cone. Most strikingly, at E12, high levels of GFP expression are found in the embryonic vessels of the placenta (Figure 1I). Thus, similar to the other major hematopoietic tissues in the embryo, the placental vasculature expresses Ly-6A GFP, suggesting a potential for hematopoietic activity.

Placenta Contains Potent HSCs Beginning at Midgestation

Previously, Alvarez-Silva et al. (2003) have found hematopoietic progenitor activity in the placenta. However, the presence of HSC activity was not tested. To examine the placenta for potent, adult-engrafting HSCs, we obtained E9–E12 embryos (human β-*globin* transgenic), dissected the placenta (without the decidua or umbilical vessels), made a single-cell suspension, and injected various doses of cells (placental tissue equivalents) into irradiated, adult recipients. Engraftment was tested by peripheral blood DNA PCR for the presence of the donor (human β-*globin*) marker at 1 and greater than 4 months posttransplantation. As shown in Table 1, no engrafted mice were found after transplantation of E9 or E10 placenta cells. However, potent engraftment (greater than 10% donor cell contribution) was found beginning at E11 and was present at high levels at E12. We tested these recipients for multilineage engraftment. High-level engraftment was observed in all hematopoietic tissues and in sorted myeloid, T lymphoid, and B lymphoid cells in recipients of E11 (not shown) and E12 placental cells (Figure 2A). Moreover, the transplantation of the bone marrow from these primary recipients into secondary, adult, irradiated recipients resulted in similar high-level repopulation at greater than 4 months posttransplantation (six positive/six injected, range of repopulation: 75%–98%). Frequency analysis of HSCs within the E12 placenta showed 1 HSC per 49,713 cells, with approximately 12 HSCs per placenta (as determined by Poisson statistics). Thus, the placenta contains potent repopulating cells that fulfill all the established functional criteria of HSCs. However, in contrast to the AGM, we found that HSC numbers in the placenta were not expanded during an organ culture step (data not shown). This is most likely due to the large size of the placenta (compared to the AGM), making it unsuitable for explant culture. Thus, while the placenta contains HSCs, it is uncertain whether the placenta possesses the intrinsic ability to generate and expand HSCs.

All Placental HSCs Are Contained within the GFP$^+$ Fraction

We next performed flow cytometric analysis to determine the number and phenotypic characteristics of GFP$^+$ cells in the midgestation placenta. E12 placenta cells were stained with antibodies specific for CD31 (endothelial, macrophage and AGM HSC marker [North et al., 2002]), CD34 (endothelial and AGM HSC marker [Sanchez et al., 1996]), c-kit (HSC and immature, hematopoietic progenitor marker [Sanchez et al., 1996]), CD45 (pan-hematopietic marker [Morrison et al., 1997]), Ter119 (erythroid progenitor marker [Kina et al., 2000]), and CD41 (hematopoietic progenitor and megakaryo-

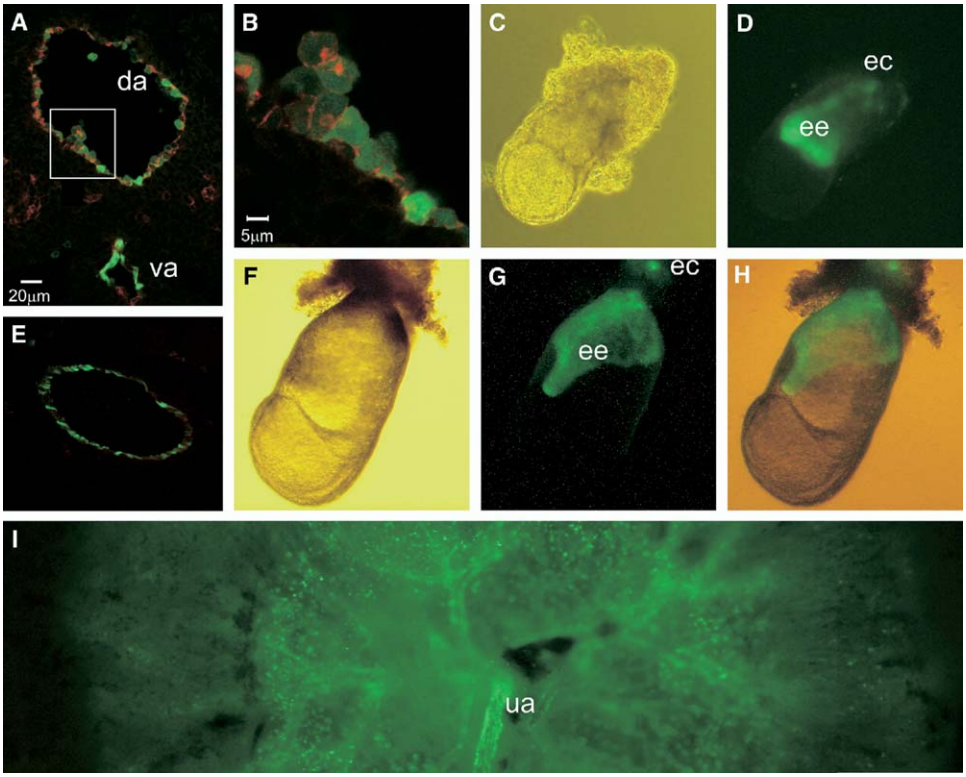

Figure 1. Expression of Ly-6A GFP during Placental Development

(A–I) (A, B, and E) Fluorescent microscopic examination of cryosections, (C, D, F–H) whole embryos, and (I) whole placenta was performed. (A) Transverse section through E11 Ly-6A GFP aorta and vitelline artery stained with anti-CD34 antibody. (B) Close-up view of hematopoietic cluster (boxed area in [A]). (E) Transverse section through E10 *Ly-6A GFP* umbilical artery stained with anti-CD31 antibody. (C and D) (C) Brightfield and (D) fluorescent light view of a whole prestreak E6 *Ly-6A GFP* conceptus. (F) Brightfield, (G) fluorescent, and (H) merged views of a whole E7.25 *Ly-6A GFP* conceptus. (I) Coronal view of the embryonic face of an E12 *Ly-6A GFP* placenta showing the GFP fluorescence in the umbilical artery and the embryonic vessels of the labyrinth. GFP, green fluorescence; CD31/CD34, red fluorescence; ee, extraembryonic ectoderm; ec, ectoplacental cone; ua, umbilical artery; da, dorsal aorta; va, vitelline artery. In (A), ventral is down.

cyte marker [Mikkola et al., 2003]). GFP$^+$ cells (Figure 2B) were found to represent 3.09% of the total viable, nucleated cell population (absolute number per placenta = 1.8 × 10^4 GFP$^+$ cells). No GFP$^+$ cells were detected in the maternally derived decidua (Figure 2C). This was due to the paternal transmission of the transgene and confirms the embryonic origins of GFP$^+$ cells. In addition, when E12 decidua cells were transplanted, no HSC activity was detected (1 month posttranslation). Not surprisingly, the placenta is 36% erythroid, as determined by Ter119 staining (Figure 2H), and appears to have a high endothelial cell content, as judged by the fact that 40% of placental cells are CD31$^+$ (Figure 2D). However, CD31 also marks cells of the trophoblast lineage (Cross et al., 2003a). Nonerythroid hematopoietic cells make up 5.7%–7.6% of the placenta (CD41- and CD45-positive cells; Figures 2I and 2E, respectively). There is almost no overlap in Ter119 and Ly-6A GFP expression. However, 7%, 13%, 15%, and 78% of the respective CD31$^+$, c-kit$^+$, CD45$^+$, and CD34$^+$ populations are Ly-6A GFP$^+$. Most interestingly, 75%, 66%, and 56% of GFP$^+$ cells express CD31, c-kit (Figure 2F), and CD34 (Figure 2G), respectively.

Since AGM HSCs coexpress c-kit and CD34, we tested the overlap in expression of these markers with GFP in placental cells. As shown in Table 2, over 50% of GFP$^+$ cells are c-kit$^+$CD34$^+$. Similarly, over 50% of GFP$^+$ cells are c-kit$^+$CD31$^+$, while lower percentages of GFP$^+$ cells are c-kit$^+$CD41$^+$ and c-kit$^+$CD45$^+$. This marker distribution within the GFP$^+$ placental cell population is reminiscent of that observed for AGM HSCs (de Bruijn et al., 2002) and, thus, suggests that at least some of the placental GFP$^+$ cells are HSCs.

To determine whether the placental HSC activity lies within the GFP$^+$ fraction of cells, we sorted GFP$^+$ and GFP$^-$ cells from E12 transgenic placentas. One and 0.25 placenta equivalents of cells were injected into irradiated, adult recipients and were analyzed 1 and/or 4 months later for donor cell engraftment. As shown in Table 1, all HSC activity was found in the GFP$^+$ fraction. These cells yielded high-level, long-term repopulation and contributed to all hematopoietic lineages (not shown). In addition, secondary transplantations of bone marrow showed that GFP$^+$ placental HSCs are selfrenewing (four positive/six injected, 1 month posttransplantation). Thus, all placental HSCs are Ly-6A GFP expressing. However, since frequency analysis demonstrates that there are only approximately 12 HSCs per

Table 1. Adult-Repopulating HSC Activity in the Placenta at Different Developmental Stages

Stage	Placenta Equivalent	Number of Mice Repopulated/Total Transplanted	Range of Repopulation
E9	2–4	0/3	0
E10 < 35 SP	3	0/2	0
> 35 SP	1–3	0/4	0
E11	0.5–3	1/5	100%
E12	1	3/3	15%–62%
	0.3	3/4	33%–61%
	0.1	3/6	48%–67%
E12 GFP$^+$	1	3/6	28%–100%
	0.25	4/9	32%–100%
E12 GFP$^-$	1	0/6	0
	0.25	0/8	0

Placentas were isolated from either human *β-globin* or from *Ly-6A GFP* transgenic mice, dissected free of deciduas and umbilical vessels, dissociated by collagenase treatment, and injected into irradiated, adult recipients. The amount of injected material is indicated in placenta tissue equivalents. At greater than 1 and/or 4 months, peripheral blood was collected and tested for the presence of the donor marker by semiquantitative PCR. The number of repopulated mice (>10% donor contribution) per total number of injected mice is indicated with the corresponding percentages of repopulation. E, embryonic day; SP, somite pairs.

Table 2. Multicolor Flow Cytometric Analysis of E12 Placental Cells

	% of Total	% of GFP$^+$	Absolute Number/Placenta
GFP$^+$	2.54 ± 0.47		15216 ± 2809
GFP$^+$c-kit$^+$CD34$^+$		54.70 ± 11.27	7958 ± 1429
GFP$^+$c-kit$^+$CD31$^+$		55.26 ± 16.74	8654 ± 3237
GFP$^+$c-kit$^+$CD41$^+$		32.62 ± 7.43	5044 ± 1508
GFP$^+$c-kit$^+$CD45$^+$		11.88 ± 2.32	1743 ± 424

Placentas were isolated from E12 *Ly-6a GFP* transgenic embryos, dissected free of deciduas and umbilical vessels, dissociated by collagenase treatment, and stained with c-kit-specific antibody (allophycocyanin conjugated) and CD34-, CD31-, CD41-, or CD45-specific antibody (phycoerythrin conjugated). Dead cells were excluded by 7AAD staining. GFP$^+$ cells were gated and further analyzed for coexpression of the above-listed markers.

E12 placenta, not all GFP$^+$ cells are HSCs. These results are consistent with previous findings in the AGM region for GFP$^+$ cells.

GFP$^+$ Cells Localize to Vascular Labyrinth

Since flow cytometric analysis demonstrated that GFP$^+$ cells were distributed between the cell fractions characterized by both endothelial and hematopoietic stem/progenitor markers, we took a temporal and spatial immunostaining approach to examine the specific localization of these cells. We stained *Ly-6A GFP* transgenic placentas from E9 (not shown) and E10–E12 embryos (Figure 3) with antibodies specific for CD31, CD34, and CD41. For orientation purposes, we show a schematic diagram of a transverse and coronal section of the placenta (Figure 3P). Distinct differences in the expression patterns of the three surface markers were clearly observed in both coronal sections (Figures 3M–3O) and transverse sections (Figures 3A–3C, 3E–3G, and 3I–3K). CD31 is expressed in many cells of the outer placenta spongiotrophoblast layer (Figures 3A–3C and 3M). It is highly expressed on the cells of the dilated maternal blood vessels and at a lower level on endothelial cells in the labyrinth. It is also expressed by a few endothelial cells lining the vessels in the chorionic plate (Figure 3D). Expression begins in the outer layer at E9 and by E12 is found also in the inner placenta, although at lower levels. CD34 is expressed exclusively in the cells of the inner placenta (Figures 3E–3G, 3N), where it seems to outline the embryonic vessels (Figure 3H). Expression is low at E9 and E10 and increases thereafter. In complete contrast to CD31 and CD34, CD41 shows a punctate expression pattern

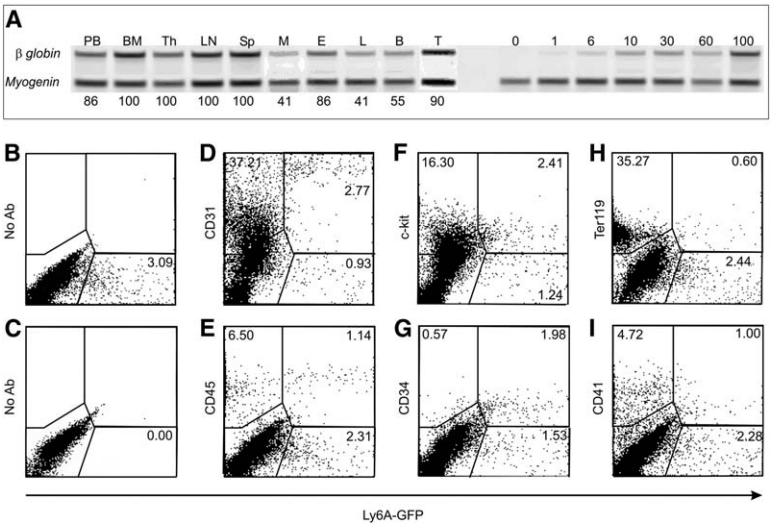

Figure 2. Multilineage Analysis of Mouse Reconstituted with Placenta-Derived HSCs and Flow Cytometric Analysis of E12 *Ly-6A GFP* Placental Cells

(A) DNA PCR multilineage contribution analysis is shown for an adult, irradiated recipient transplanted with 0.3 embryo equivalents of E12 placenta cells (human *β globin* transgenic). Genomic DNA was obtained from organs and sorted cell hematopoietic populations at greater than 4 months post-transplantation. DNA was analyzed for the presence of the donor marker (*β globin*) by semiquantitative PCR. The percentage of donor contribution (along the bottom of the panel) was calculated from the ratios between the donor marker and myogenin and by comparison with a standard curve generated from control mixes of DNA ranging from 0% to 100% donor marker. PB, peripheral blood; BM, bone marrow; Th, thymus; LN, lymph node; Sp, spleen; M, sorted BM myeloid cells; E, sorted BM erythroid cells; L, sorted BM lymphoid cells; B, sorted spleen B lymphocytes; T, sorted spleen T lymphocytes.

(B–I) Dissociated cells from (B, D–I) E12 *Ly-6A GFP* transgenic placentas or (C) decidua were stained with phycoerythrin-conjugated antibodies against CD31, CD45, c-kit, CD34, Ter119, and CD41 as indicated on the y axis. GFP fluorescence is shown on the x axis. Percentages for each population are indicated inside the quadrants. Dead cells were excluded by gating out 7AAD-positive cells.

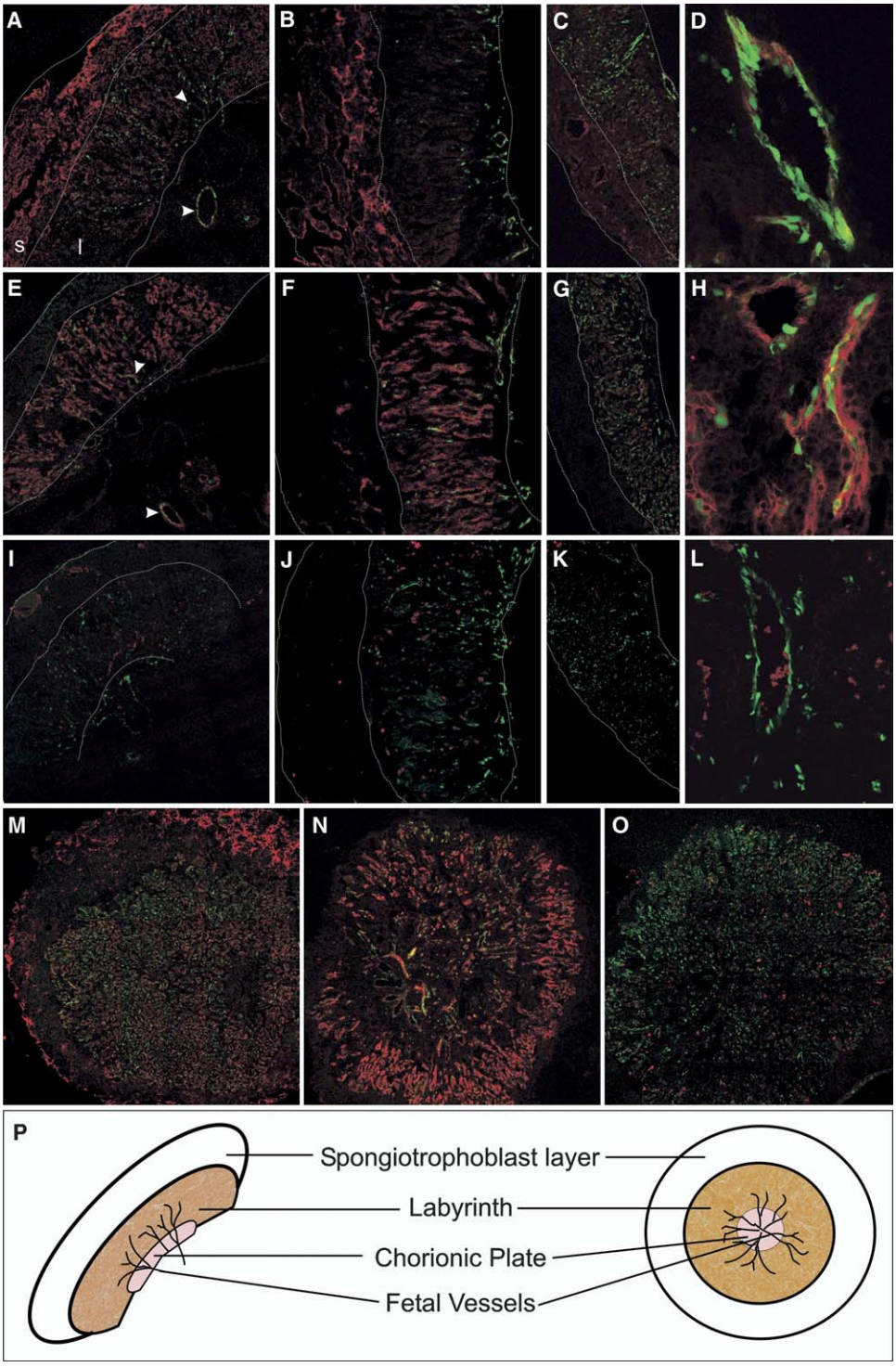

Figure 3. Colocalization of Expression of Ly-6A GFP with CD31, CD34, and CD41

(A–P) Placentas from (A, E, and I) E10, (B, F, and J) E11, and (C, G, and K) E12 *Ly-6A GFP* embryos were cryosectioned at 10 μm and stained with antibodies against (A–C) CD31, (E–G) CD34, and (I–K) CD41. (P) A schematic view of a transverse (left) and coronal (right) section of placenta. High magnification of vasculature and blood cells in *Ly-6A GFP* cryosections. (D) E12 section stained with anti-CD31 antibody. (H) E11 section stained with anti-CD34 antibody. (L) E11 section stained with anti-CD41 antibody. Coronal sections of E12 *Ly-6A GFP* placentas stained with (M) anti-CD31 antibody, (N) anti-CD34 antibody, and (O) anti-CD41 antibody. (A–C, E–G, I–K, M–O) Sections were analyzed by confocal microscopy, and individual pictures (tiles) were assembled to display the entire structure. In the E10 panels, the intensity of the red channel was enhanced to reveal placental morphology. The boundaries of the labyrinth and spongiotrophoblast regions in panels (A)–(C), (E)–(G), and (I)–(K) are highlighted with dotted lines. Arrowheads indicate the embryonic vessels. Green, GFP; red, antibody stainings; s, spongiotrophoblast layer; l, labyrinth.

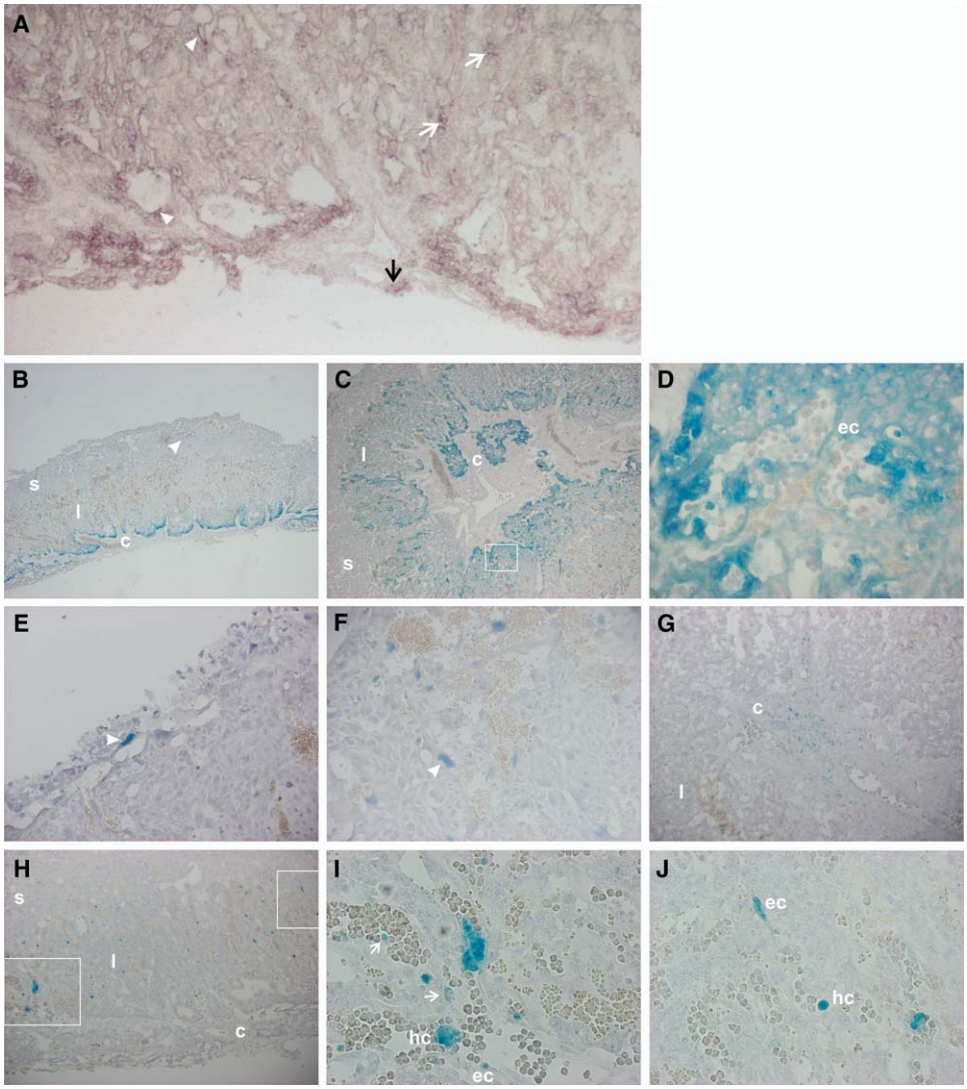

Figure 4. Expression of c-kit in the E12 Placenta and Hematopoietic Transcription Factors in the E11 Placenta

(A) E12 placentas were cryosectioned at 10 μm and hybridized with a riboprobe for *c-kit*. Shown is a 10× magnification of a transverse section; the embryonic face of the placenta is oriented downward. *c-kit* expression is found in endothelial cells (arrowheads), and in mesenchymal cells in the chorionic plate (black arrow), and in the labyrinth (white arrows).

(B–J) Placentas from (B–D) *GATA2 LacZ*, (E and F) *GATA3*$^{lz/+}$, and (G–J) *Runx1*$^{lz/+}$ E11 embryos were stained for β-galactosidase and cryosectioned at 10 μm. Sections were counterstained with hematoxylin and inspected by light microscopy. (B, E, and H–J) Transverse sections. (C, D, F, and G) Coronal sections. Magnifications: (B and C) 4×, (G and H) 10×, (E and F) 20×, (D, I, and J) 40×. (D) is the boxed region in (C). (I) and (J) are close-up views of the boxed areas in (H). Arrowheads indicate trophoblast giant cells, and arrows indicate cells underlying the endothelium in a region in which cells may be budding into the lumen of the vessels. s, spongiotrophoblast layer; l, labyrinth; c, chorionic plate; hc, hematopoietic cell; ec, endothelial cell.

mainly in the inner placenta (Figures 3I–3K, 3O), where it predominantly marks cells within the blood vessels (Figure 3L).

In situ hybridization was performed to localize *c-kit*-expressing cells in the placenta, since high background staining was observed with c-kit-specific antibodies. As shown in Figure 4A, *c-kit* expression is found in mesenchymal cells of the chorionic plate (black arrow) and in islands of mesenchymal cells in the labyrinth (white arrows). Many of the vessels also contained *c-kit*-expressing endothelial cells (arrowheads). We also detected expression in some trophoblast giant cells (not shown). The expression of *c-kit* was highest on the embryonic face of the placenta.

The general expression pattern of Ly-6A GFP is very similar to that of CD34, as expected from the results of the flow cytometric analysis (78% of CD34$^+$ cells are GFP$^+$). Expression begins at E9 in some of the cells of the labyrinth and increases thereafter. GFP$^+$ cells are also found lining the fetal blood vessels in the chorionic plate and in the umbilical vessel (arrowheads in Figures 3A and 3E). Most of the overlap in the expression of GFP with CD34 is in the labyrinth region (Figure 3F) in the endothelial cells lining the fetal vessels that form a

network through this region. There appears to be only a small amount of overlap of CD31 with GFP-expressing cells, confirming flow cytometric data (only 7% of $CD31^+$ cells are GFP^+). These cells are found in the large embryonic vessels within the chorionic plate and also in the umbilical artery (Figures 3A and 3D and Figure 1E). We observed no overlap in the expression of CD41 and GFP (Figures 3J and 3L). Thus, these immunostaining and in situ transcription results taken together with the multicolor flow cytometric analysis and the finding that all HSCs are GFP^+ strongly suggest that placental HSCs are localized within the endothelium of the embryonic vessels in the chorionic and labyrinth regions.

Placental Embryonic Vessel Endothelium Expresses Hematopoietic Transcription Factors

To further explore whether HSCs in the placenta may be generated in situ, we examined the expression pattern of three hematopoietic transcription factors, *GATA2*, *GATA3*, and *Runx1*, known to be important for HSC/progenitor development. Briefly, embryos deficient for *GATA2* die at E10.5, display severe anemia, and lack HSCs (Tsai et al., 1994). A haploid dose of *GATA2* results in defective HSC expansion in the AGM (Ling et al., 2004). The disruption of the related transcription factor *GATA3* leaves YS erythropoiesis largely unaffected, but results in defective fetal liver hematopoiesis, as demonstrated by a reduction in hematopoietic colony formation (Pandolfi et al., 1995). *Runx1* deficiency results in E12.5 lethality, fetal liver anemia, and an absence of HSCs (Okuda et al., 1996; Wang et al., 1996). A haploid dose of *Runx1* disrupts the normal pattern of HSC emergence in the embryo (Cai et al., 2000).

We obtained E11 *GATA2 lacZ*, *GATA3 lacZ*, and *Runx1 lacZ* embryos and stained and sectioned the placentas. *GATA2 lacZ* embryos carry a *lacZ* reporter transgene that recapitulates the endogenous *GATA2* gene expression pattern (Zhou et al., 1998). *GATA3 lacZ* and *Runx1 lacZ* embryos contain an endogenous gene-targeted *lacZ* reporter (North et al., 1999; van Doorninck et al., 1999).

The expression pattern of all three transcription factors differed. In *GATA2 lacZ* transgenic placental sections, we found some expression in trophoblast giant cells, as reported previously (arrowhead in Figure 4B). However, higher levels of β-galactosidase staining were found in the labyrinth (Figures 4B and 4C). An increasing intensity of staining was observed toward the fetal side of the placenta, especially on the borders of the labyrinth region with the chorionic plate. *GATA2* is expressed in some endothelial cells and in the underlying cells that surround the fetal blood vessels (Figure 4D). Like *GATA2*, *GATA3* is expressed in the trophoblast giant cells, although to higher levels (Figures 4E and 4F, arrowheads). In contrast to *GATA2*, *GATA3* expression is restricted to only these few cells at the fetal-maternal interface. This pattern of expression confirms the previous pattern seen by others using in situ transcription analysis (Ng et al., 1994). The endothelial expression of *GATA2* (but not *GATA3*) is reminiscent of the expression of *GATA2* at the onset of HSC emergence in the midgestation aorta (Minegishi et al., 1999; Zhou et al., 1998).

Runx1 expression appears to localize to cells within the blood vessels of the labyrinth (in the circulation, as well as cells attached to the lumenal side of the endothelium), to endothelial cells (Figures 4H–4J), and to cells located just underneath the endothelium (arrows in Figure 4I). Occasionally, we see clusters of β-galactosidase-positive cells within the circulation or attached to the endothelium. There also seems to be an accumulation of positive cells in the chorionic plate (Figure 4G). These *Runx1*-expressing cells are located both within the walls of the vessels and surrounding the major blood vessels at their junction with the umbilical vessels (Figure 4G). Thus, the distribution of *Runx1*-expressing cells amongst hematopoietic, endothelial, and mesenchymal cells is similar to what has been reported for *Runx1*-expressing cells in the AGM and suggests that Runx1 may also be involved in the generation of HSCs in the placenta (North et al., 1999; North et al., 2002).

Discussion

We have shown here that the placenta, a highly vascularized tissue necessary for the exchange of oxygen and nutrients between the embryo and the mother, contains potent, adult-repopulating HSCs. These are not maternally derived HSCs, but are derived from the conceptus, as evidenced by the presence of the human *β-globin* and *Ly-6A GFP* transgenic markers. They are as potent as HSCs derived from the adult bone marrow in that they give rise to long-term, high-level (15%–100%), multilineage hematopoietic engraftment of irradiated, adult recipients. Placental HSCs are also selfrenewing, since they can repopulate secondary recipients. These data now raise the possibility that, along with the AGM and YS, the placenta may be an additional generating source of HSCs that sequentially migrate and colonize the fetal liver and bone marrow (Figure 5).

The Placenta as a Reservoir for Expanding HSCs or for Their Generation?

Our transplantation data show that prior to E11, placental HSC activity appears to be limiting. We found no HSCs in the placenta at E9 or E10. At E11, only one out of five transplanted recipients receiving two tissue equivalents of placental cells is HSC engrafted. At E12, we found 12 HSCs per placenta. In more recent experiments (not shown), we found that HSC numbers in the placenta begin to decrease at E13 and disappear thereafter. Thus, the numbers of HSCs in the placenta increase rapidly with developmental time and peak at E12–E13. Indeed, large numbers of immature progenitors have been found previously in the placenta and greatly exceed the numbers found in the fetal liver (Alvarez-Silva et al., 2003). Others have shown that the human placenta secretes hematopoietic growth factors that stimulate hematopoietic colony formation (Burgess et al., 1977). More recent work demonstrates that mesenchymal progenitor cells isolated from the human placenta can expand long-term, culture-initiating cells from cord blood (Zhang et al., 2004). Hence, the he-

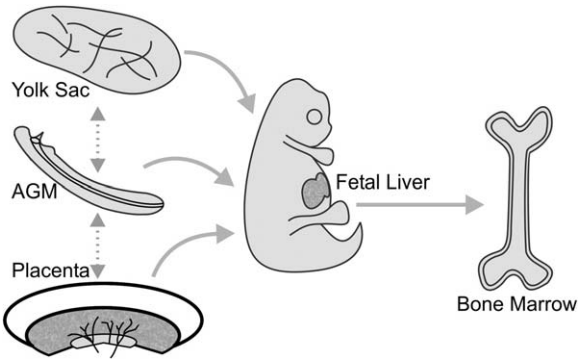

Figure 5. HSC Dissemination during Development

Model for HSC generation and migration. HSCs may be generated and/or expanded in up to three different anatomical sites during mouse development: YS, AGM, and placenta. Some cross-seeding may occur between these sites via the circulation (dashed arrows). HSCs from all three sources may be needed to colonize the fetal liver (curved, solid arrows), which remains the main location of hematopoiesis until birth, when hematopoiesis permanently relocates to the bone marrow for the entire adult life.

matopoietic growth capacity of the placenta is enormous, and it is not surprising that it is a potent hematopoietic microenvironment for HSCs.

Recently, quantitative data on the numbers of HSCs present in the various hematopoietic tissues and circulation in the midgestation mouse have been presented. Kumaravelu et al. (2002) found that at E12 there are 3.2 HSCs in circulation. Correcting for numbers of HSCs in tissues due to circulation, the AGM was found to contain 2.7 HSCs, the YS 1.8, the liver 53, and the umbilical cord 0.8. Given that the E12 placenta contains 12 HSCs (four times more HSCs than in the whole of the embryonic blood), and considering that the fetal liver contains 53 HSCs at E12, it is highly likely that the placenta is a potent HSC contributor to the colonization of the fetal liver, along with the AGM and YS.

It is as yet uncertain whether the HSCs in the placenta are intrinsically or extrinsically generated. If the placenta was found to contain HSCs at E10 or earlier, it would implicate the placenta as the first site of emergence of HSCs in the mouse embryo. Previously, to detect the onset of HSC activity in the E10 AGM region, it was necessary to transplant 96 adult recipients with a total of 112 AGM tissue equivalents of cells to observe the long-term, high-level, multilineage repopulation of 3 recipients (Muller et al., 1994). Given the limited number of mice transplanted with E10 placental cells in this study, it remains a possibility that the placenta contains HSCs at this earlier time point. To address the issue of autonomous generation, we performed explant cultures with whole placentas. While we could maintain HSCs in AGM explants, no HSCs could be maintained in placentas. Thus, the origins of the HSCs in the placenta are unclear and await the results of lineage-marking approaches.

Association of Placental HSCs and the Vasculature

The strong association of hematopoietic and endothelial markers on the earliest hematopoietic cells in the mouse embryo, including HSCs, prompted our studies on the placenta. The *Ly-6A GFP* transgenic marker was found to be expressed in the endothelial cells lining the major vessels of the placenta. Moreover, all HSC activity was attributed to the GFP^+ fraction of the placenta. Thus, consistent with previous data, the Ly-6A GFP expression again marks all HSCs. While the number of GFP^+ cells in the placenta far exceeds the number of HSCs, other markers (such as CD34, CD31, c-kit, etc.) were used to further localize placental HSCs. Previous studies on the AGM have shown that all AGM HSCs are $c\text{-}kit^+CD34^+$ (Sanchez et al., 1996) and $CD31^+$ (North et al., 2002). Our multicolor flow cytometric analysis strongly suggests that placental HSCs are $GFP^+c\text{-}kit^+CD34^+$.

Immunostaining of *Ly-6A GFP* transgenic placentas has further supported the notion that these HSCs are $CD34^+$. CD34- and GFP-coexpressing cells were found in the labyrinth region. This highly vascularized region showed coexpressing cells lining the embryonic vessels. Similarly, although at a lower frequency, CD31- and GFP-coexpressing cells were also found in endothelial cells lining the vessels of the labyrinth. Moreover, *c-kit* expression was found in a few cells in this region by in situ transcription analysis. In no sections did we find prominent hematopoietic clusters with these phenotypic characteristics. However, we did observe rare single GFP^+ cells adhering closely to the lumenal surface of the larger vessels. Thus, in analogy to the AGM region in which GFP and CD34/CD31 are coexpressed in the ventral aortic endothelial cells and some cells of the associated hematopoietic clusters, these markers may also indicate associations of these two lineages in putative hemogenic endothelium of the placenta.

A Role for Transcription Factors GATA2 and Runx1 in Placental Hematopoiesis?

Previously, it was reported that *GATA2* and *GATA3* are highly expressed in the placenta in the trophoblast giant cells positioned at the embryonic-maternal interface during midgestation (Ng et al., 1994). The GATA transcription factors regulate the expression of a number of trophoblast-specific genes, such as the prolactin hormone placenta lactogen I and the angiogenic factor proliferin (Ma et al., 1997; Ng et al., 1994). These molecules appear to play an important role in the neovascularization of the placenta in the interface region (Ma et al., 1997). We also found expression of these transcription factors in trophoblast giant cells. The lower frequency of *GATA3*- expressing trophoblast cells that we found most likely was a reflection of a haploinsufficiency of *GATA3* due to a defective targeted allele. Nonetheless, *GATA3* was restricted in its expression to these cells and is known to rapidly decline in its expression after E10 (Ng et al., 1994).

GATA2 had a much more widespread expression pattern than previously described. *GATA2* was expressed in some endothelial cells lining the vessels of the labyrinth, as well as in many cells surrounding the vessels. The intensity of expression increased with proximity to the chorionic plate of the placenta. At the interface of the labyrinth region with the chorionic plate, *GATA2* expression was the highest. Thus, GATA2

may be required for the neovascularization of the labyrinth by regulating the expression of angiogenic factors. Additionally, it may also be involved in the generation and/or proliferation of HSCs from hemogenic endothelial cells in the labyrinth.

Previously, the expression of *Runx1* in the placenta was found by RT-PCR (Alvarez-Silva et al., 2003), but its expression pattern within the placenta was not explored. As one of the most important HSC transcription factors, Runx1 is required for the emergence of HSCs in the midgestation embryo and is expressed in aortic hematopoietic clusters, endothelial cells, and underlying mesenchymal cells (North et al., 1999). The expression pattern of *Runx1 lacZ* in the placenta is reminiscent of this pattern. We found high-level *Runx1*-expressing cells in the lumen of the labyrinth vasculature as well as in some of the endothelial cells lining the vessels. Lower-level expressing cells were found underlying some of the endothelium. Thus, as in the AGM region, Runx1 is most likely playing a role in the emergence of HSCs. We are currently testing E12 placentas from *Runx1*$^{+/-}$ and *Runx1*$^{-/-}$ embryos for HSC activity in transplantation assays.

Finally, we have also investigated the possible role of the oncogene c-*fos* in placental HSC generation. High levels of c-*fos* have previously been observed in the placenta (Muller et al., 1983), and a placental defect in *c-fos*$^{-/-}$ embryos has been suggested on the basis of lower placenta weight, although no conclusive evidence had been provided (Johnson et al., 1992). We transplanted E12 placentas from *c-fos*$^{-/-}$ embryos and found some low-level HSC activity still present (data not shown). However, a more detailed analysis is required to unveil possible subtle differences in HSC frequency between wild-type and *c-fos*$^{-/-}$ placentas.

In summary, we have found that the placenta is a highly hematopoietic tissue, supporting the growth and/or emergence of HSCs. As found by flow cytometric and histologic analysis, it appears to possess hemogenic endothelium, and we will test this in future experiments. Notwithstanding, this may be taken as another example of the recurring theme of HSC development within the major vasculature of the embryo and may indicate that the *Ly-6A GFP* transgene serves as a useful marker for hemogenic endothelium in the embryo. Thus, our results now add the placenta to the list of embryonic tissues that contain HSCs (Figure 5), and we propose that it plays an important role in the long-term development of the adult hematopoietic system and the colonization of the bone marrow with HSCs.

Experimental Procedures

Embryo Generation

Timed matings were set up between male *Ly-6A GFP* (de Bruijn et al., 2002) or line 72 human β-*globin* (Strouboulis et al., 1992) transgenic mice and wild-type (C57BL/10 × CBA)F_1 females; between male transgenic *GATA-2 YAC d16Z* mice (Zhou et al., 1998) and wild-type C57BL/6 females; and between male knockin *Cbfa2*$^{lz/+}$ (North et al., 1999) or *GATA-3*$^{lz/+}$ mice and (C57BL/10 × CBA)F_1 females. The day of vaginal plug detection was considered as embryonic day 0. For embryo generation, the transgene was always inherited through the male parent in order to avoid contribution by genetically marked maternal cells in transplantation assays. Animals were housed according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals.

Cell Preparations

Pregnant mice were killed by cervical dislocation. Placentas were dissected (free of umbilical cord and maternal decidua) and treated for 1.5 hr at 37°C with 0.125% type I collagenase (SIGMA-ALDRICH CHEMIE Gmbh, Germany) in PBS supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells were dispersed, washed, and resuspended either in PBS for transplantation or in PBS supplemented with 10% fetal calf serum and penicillin/streptomycin for cell sorting or flow cytometric analysis. In some cases, ficoll gradients (Lymphoprep, Axis-Shield, Oslo, Norway) were used to remove the large number of erythroid cells present in placental preparations prior to cell sorting.

Analysis of Long-Term, Multilineage Repopulating Activity

The presence of definitive HSCs in placental cell suspensions was determined by intravenous injection into irradiated, adult recipients as described previously (de Bruijn and Dzierzak, 2002). Recipient (C57BL/10 × CBA)F_1 males or females received a split radiation dose of 900 rad from a ^{137}Cs source. To ensure short-term survival, 2×10^5 adult (C57BL/10 × CBA)F_1 spleen cells were coinjected. One and/or 4 months posttransplantation, peripheral blood DNA samples were analyzed for donor cell contribution by PCR as described previously (de Bruijn and Dzierzak, 2002; de Bruijn et al., 2002). For Poisson statistical analysis, the number of total cells injected (x axis) was plotted against the percentage of mice negative for repopulation (y axis). The resulting straight line had a gradient of $y = 100e^{-2E-05x}$. The frequency of HSCs is by definition determined at 37% of negative mice. In E12 placenta, the frequency of HSCs was calculated to be 1 in 49,713 cells and, thus, 12 HSCs per E12 placenta (6×10^5 nucleated cells per placenta).

For the analysis of multilineage repopulation, thymus, lymph nodes, bone marrow, spleen, and peripheral blood samples were collected from repopulated animals, DNA was isolated, and donor contribution was assessed by PCR. Cells of the T, B, erythroid, and myeloid lineages were sorted by using a combination of antibodies listed below, and the DNA was analyzed for the presence of donor markers. A total of 2–3 × 10^6 cells from the bone marrow of repopulated mice were injected into secondary recipients to assess selfrenewal capacity.

Cell Sorting and Flow Cytometry Analysis

All antibody stainings were carried out in a volume of 100–200 μl in PBS/10% fetal calf serum on ice for 30 min with the following antibodies (PharMingen): anti-CD4-PE (clone GK1.5), anti-CD8a-PE (clone 53-6.7), anti-CD31-PE (clone MEC 13.3), anti-B220-FITC (clone RA3-6B2), anti-Ly6C-FITC, anti-Ter119-PE (clone TER-119), anti-CD45-PE (clone 30-F11), anti-CD34-PE (clone RAM34), anti-c-kit-PE (clone 2B8), anti-c-kit-APC (clone 2B8), anti-CD41-PE (clone MwReg30), anti-Gr-1-PE (clone RB6-8C5), and anti-Mac1-PE (clone M1/70). Just before analysis, 7AAD (7-aminoactinomycin D, Molecular Probes, Leiden, NL) was added to allow live/dead cell discrimination. Cells were sorted on a FACSVantage (BD Biosciences), and analysis was carried out on a FACScan (BD Biosciences). For single and double antibody-stained samples of *Ly-6A GFP* placental cells, 5×10^4 and 1×10^6 events, respectively, were analyzed on the flow cytometer, and CellQuest (BD Biosciences) was used for data presentation.

Immunohistochemistry and Confocal Microscopy

Dissected placentas were fixed for 1–2 hr, rotated in 2% paraformaldehyde/PBS at 4°C, and equilibrated overnight in 30% sucrose/PBS at 4°C. Fixed tissues were embedded in Tissue Tek and quick-frozen on dry ice, and 10 μm cryosections were prepared. Immunohistochemical stainings were carried out as previously described (de Bruijn et al., 2002) by using biotin-conjugated anti-CD31 (clone MEC13.3; PharMingen), biotin-conjugated anti-CD34 (clone RAM34; PharMingen), purified anti-CD41 (clone MWReg30; PharMingen), biotin-conjugated anti-rat IgG1 (clone RG11/39.4; PharMingen), and Streptavidin-Cy5 (Rockland Immunochemicals, Inc., Gilberts-

ville, PS). Antibody stainings were detected by laser scanning microscopy.

In Situ Hybridization

A 300 bp product was obtained by RT-PCR with E12 placenta RNA and mouse c-kit-specific primers 5′-AAGTGTACCCCGTAGAC AGG-3′ and 5′-TGGTGTGGCCCCTTAAGTAC-3′ (region 4397–4797 of accession number NM 021099), and cloned into the p-GEM-T Easy vector (Promega). Digoxigenin-labeled riboprobes were prepared by in vitro transcription by using a DIG RNA Labeling Kit (Roche). Tissues were fixed in 4% paraformaldehyde/30% sucrose/PBS at 4°C overnight, submerged in Tissue-Tek (Sakura Finetek, The Netherlands), and quick-frozen on dry ice. 10 μm sections were prepared; treated with 100 mM Glycine in PBS for 10 min at room temperature, 0.3% Triton X-100 in PBS for 15 min at room temperature, 1 μg/ml Proteinase K in TE for 30 min at 37°C; and fixed in 4% paraformaldehyde/PBS for 5 min at 4°C. Sections were then acetylated (13% v/v triethanolamine, 0.175% v/v hydrochloric acid, 0.25% v/v acetic acid) for 10 min at room temperature and incubated for 1 hr at room temperature in hybridization buffer (50%, 5× SSC, 1× Denhardt's, 0.1% Tween-20, 250 μg/ml yeast t-RNA, 500 μg/ml salmon sperm DNA). Probes were diluted 100 pg in 100 μl hybridization buffer, denatured for 5 min at 80°C, and incubated with the sections overnight at 65°C. After extensive washing with 0.2× SSC, slides were blocked with 10% heat-inactivated sheep serum in 0.1 M Tris (pH 7.5)/0.15 M NaCl all day at room temperature and incubated overnight with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:4000, Roche) at 4°C. Sections were then incubated with 2% NBT/BCIP solution (Roche) in 0.1 M Tris (pH 9.5)/0.1 M NaCl/50 mM $MgCl_2$/0.24 mg/ml levamisole at room temperature for 1 day. Slides were washed with TE and water, and the sections were fixed with 4% paraformaldehyde for 30 min at room temperature, washed with TE and water, and mounted with Aquamount (BDH).

LacZ Stainings

Dissected placentas were rinsed in PBS/0.02% NP40 and fixed at 4°C for 1 hr with 10% Formal Saline/0.2% Glutaraldehyde/2 mM $MgCl_2$/5 mM EGTA/0.02% NP40 (all in PBS). Tissues were then washed three times for 30 min per wash at room temperature with PBS/0.02% NP40 and stained overnight at room temperature with 5 mM $K_3Fe(CN)_6$/5 mM $K_4Fe(CN)_6$/2 mM $MgCl_2$/0.01% Nadeoxycholate/0.02% NP40/0.1% X-gal (all in PBS). The next day, tissues were washed five times with PBS and fixed, frozen, and sectioned as described above.

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